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Original communication

Assessment of viable bacteria and bacterial DNA in blood and bloodstain specimens stored under various conditions

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ABSTRACT

Microbial forensic specimens that are collected at biocrime and bioterrorism scenes include blood, tissue, cloths containing biological fluids, swabs, water, soil, and aerosols. It is preferable that pathogens in such specimens are alive and kept in a steady state. Specimens may be stored for a prolonged period before analysis; therefore, it is important to understand the effect of the storage conditions on the pathogens contained within the specimens. In this study, we prepared blood and bloodstain specimens containing Gram-negative or -positive bacteria, stored the samples for 482 days under various conditions, and measured viable bacterial counts and total bacterial contents in the samples. Viable bacteria were preserved well in the samples stored at −30 and −80 °C, but were diminished or undetectable in the samples stored at 4 °C and room temperature. The total bacterial content was maintained in the blood samples stored at −30 and −80 °C and in the bloodstain samples stored under all temperature conditions, but decreased in the blood samples stored at 4 °C and room temperature. This study showed that the storage conditions affected viable bacteria and bacterial DNA and that freezing and drying were significant for their long-term storage. We provide important information for the storage of microbial forensic specimens.

1. Introduction

Numerous documented examples of the use of pathogens in major conflicts or terrorism have been reported.[1] For example in the anthrax letter attacks in the United States in 2001, a dried endospore preparation of *Bacillus anthracis* was dispersed.[2–4] Microbial forensic specimens that would be collected in biocrime and bioterrorism investigations include blood, tissue, body fluids, body fluid stain, swabs, water, soil, and aerosols. Although microbiological analysis of the collected specimens should be performed as soon as practicable after an incident, the specimens may be stored for a prolonged period before analysis on procedural grounds. Even after analysis, it may be necessary to store unconsumed specimens in good condition as evidence for a prolonged period to enable future reanalysis when necessary. Also, it is anticipated that frozen storage of the specimens immediately after collection will not always be possible. Thus, we need to understand the effect of the storage conditions on the pathogens contained within the specimens.

In bacteriological analysis of forensic samples, it is preferable that the targeted bacteria in the specimens are alive and kept in a steady state. If viable bacteria are undetectable due to poor storage conditions, DNA analysis of the bacteria is necessary for their identification and quantitative determination. Although several studies have investigated the effects of storage solutions and temperatures on bacteria,[5–7] no study has examined the effect of storage conditions on the viability of bacteria and bacterial DNA contained in forensic samples.

In cases of biocrime and bioterrorism, the first scientific evidence indicating their occurrence for law enforcement may be pathogens detected from body fluid and body fluid-stained samples derived from a victim. In this study, blood and dried bloodstain specimens containing Gram-negative or -positive bacteria were prepared and stored for 482 days under various conditions. The viable bacteria counts in these models were measured by colony formation and changes in the population of each bacterium during the study period were determined. Quantitative procedures were established to examine the

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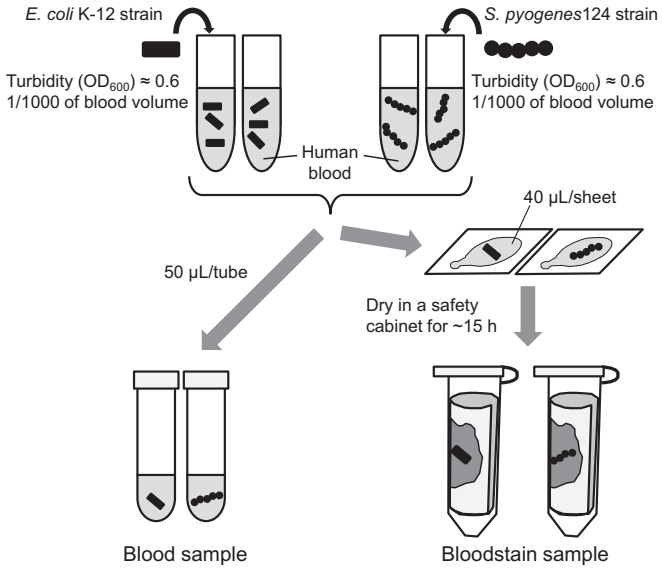

Fig. 1. Sample preparation. Blood containing *E. coli* or *S. pyogenes* was dispensed into 50-µL aliquots in screw-cap tubes and stored under various conditions. To prepare bloodstains, 40 µL of blood containing each bacterium was spotted onto a sterilized cotton cloth and air-dried at room temperature in a class II biosafety cabinet for ~15 h. The bloodstained cotton cloth was put into a 2-mL microcentrifuge tube and stored under various conditions. When the bloodstain was stored with a desiccant at room temperature, the cap of the microcentrifuge tube was left open.

bacterial DNA in the models using real-time PCR and quantitative values were compared with the viable bacteria counts, revealing the effect of the storage conditions on bacterial cells and DNA.

2. Materials and methods

2.1. Sample preparation

The Gram-negative bacterium *Escherichia coli* K-12 strain and the Gram-positive bacterium *Streptococcus pyogenes* 124 strain[8] were used in the present study. When the turbidity (OD_{600}) of each culture reached 0.6 (corresponding to 10^9–10^{10} colony forming units (CFU)/mL), a part of the culture (one-thousandth of the blood volume) was added into a blood sample that was withdrawn into a blood collection tube containing heparin sodium (Venoject II VP-H100K; Terumo, Tokyo, Japan) from a volunteer. As shown in Fig. 1, the blood containing *E. coli* or *S. pyogenes* was divided into 50-µL aliquots in screw-cap tubes and stored under the conditions described below ($n = 2$). Bloodstains were prepared as follows: 40 µL blood containing each bacterium was spotted onto a 400-mm^2 sterilized cotton cloth (approximately 15 mm in diameter) and air-dried at room temperature in a class II biosafety cabinet for ~15 h. The bloodstained cotton cloth was placed into a 2-mL microcentrifuge tube and stored under the conditions described below ($n = 2$).

2.2. Storage conditions

Blood and bloodstains containing *E. coli* or *S. pyogenes* were stored for different durations (1, 7, 14, 28, 56 (2 months), 119 (4 months), 231 (8 months), and 482 days (16 months)) at 4 temperatures (room temperature, 4 °C, −30 °C, and −80 °C). Besides normal storage at room temperature, the bloodstain samples were stored with a sheet-like desiccant (ID SHEET; AS ONE Corporation, Osaka, Japan) in a 2.5-L airtight container. The cap of each microcentrifuge tube containing a bloodstain sample was open in the airtight container.

2.3. Viable bacterial counts

Viable bacterial cells were enumerated by direct plating. The bloodstains were extracted in 2 mL sterile phosphate-buffered saline (PBS (−))with vortexing for 60 s. The blood samples and extracted bloodstain samples were diluted serially in sterile PBS (−) and spread on an agar medium plate. *E. coli* cultures were incubated under aerobic conditions at 37 °C for 24 h. *S. pyogenes* cultures were incubated under anaerobic conditions at 37 °C for 48 h. The average of two measurements was used to calculate the surviving number of cells per sample.

2.4. DNA extraction

Bacterial DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA), following the manufacturer's instructions (Appendix D: Protocols for Bacteria). Briefly, 180 µL of an enzyme solution (20 mg/mL lysozyme; 20 mM Tris–HCl, pH 8.0; 2 mM EDTA; 1.2% Triton) was put into the tube of the blood sample and then vortexed. The bloodstain sample was moistened with 180 µL of the enzyme solution in the tube and mixed well by agitation with a pipet tip. After incubation for 30 min at 37 °C, 25 µL proteinase K and 200 µL Buffer AL were added to each tube and the tubes were mixed by vortexing. After incubation for 30 min at 56 °C, 200 µL ethanol (99.5%) was added and the tubes were mixed thoroughly by vortexing. The following step was performed according to the manufacturer's instructions (Protocol: DNA Purification from Tissues) and the purified bacterial DNA was finally eluted in 100 µL Buffer AE. The DNA solution was stored at −30 °C until use. As an extraction negative control, 180 µL of the enzyme solution alone was used.

2.5. Standard DNA

E. coli and *S. pyogenes* determined viable bacteria counts were used for DNA extraction. Bacterial pellets of 5.0×10^7, 5.0×10^6, 5.0×10^5, 5.0×10^4, and 5.0×10^3 CFU were resuspended in 180 µL of the enzyme solution and bacterial DNA was eluted in 100 µL Buffer AE. These purified DNA samples were included as standards for each PCR run.

2.6. Quantification of total bacteria by real-time PCR

Total (viable and dead) bacteria were quantified with the LightCycler™ 1.1 system (Roche Diagnostics GmbH, Mannheim, Germany). The *uidA* beta-D-glucuronidase gene from *E. coli* and the *slo* streptolysin O gene from *S. pyogenes* were amplified with the primers listed in Table 1. The *uidA*-F (forward) and *uidA*-R (reverse) primers were designed previously.[9] The other primers were

Table 1
Sequences of primers used for real-time PCR.

Primer	Oligonucleotide sequence (5′ to 3′)	Position (*uidA*[a] or *slo*[b] gene)	Product length (bp)
uidA-F	TGCAACTGGACAAGGCACTA	672–691	172
uidA-R	GAACTGTTCGCCCTTCACTG	824–843	
*uidA*505-F	GTCCGCAAGGTGCACGGGAA	1250–1269	505
*uidA*505-R	TGCAGCAGAAAAGCCGCCGA	1735–1754	
*slo*211-F	ACCTGTCTCATCGAGGTGCGTA	1406–1427	211
*slo*211-R	TCCCAAGCTAAGCCGGTGCAC	1596–1616	
*slo*510-F	GCGTCGCTGGGCTACTAACGG	44–64	510
*slo*510-R	CAGCCAGCTGAAGGGCTGCT	534–553	

[a] NCBI gene ID: 946149.
[b] NCBI gene ID: 3573141.

designed using Primer-BLAST software.[10] SYBR® Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan) was used for real-time PCR. In each glass capillary tube, 2 μL extracted DNA were added to 23 μL PCR mixture containing 12.5 μL SYBR® Premix Ex Taq™ (2× concentration; containing Takara Ex Taq® HS, dNTP mixture, Mg^{2+}, and SYBR® Green I), 1 μL primer mixture (5 μM each primer), and 9.5 μL H_2O (PCR grade). The following PCR conditions were used: an initial denaturation step of 2 min at 95 °C, followed by 36 cycles of 95 °C denaturation for 10 s and 65 °C annealing and extension for 45 s. The amplification reactions and data acquisition were performed using a LightCycler™ 1.1 (Roche Diagnostics). To avoid cross-contamination, pre-PCR sample processing was performed in a separate room. To check for the amplification of human DNA, K562 DNA (Promega Corporation, Madison, WI) was used as a template for real-time PCR.

3. Results

3.1. Viable bacterial counts in blood and bloodstain samples

We determined population changes of *E. coli* and *S. pyogenes* in the blood and bloodstain samples stored for 482 days. In the blood samples stored at −30 and −80 °C, there was no significant reduction of *E. coli* and *S. pyogenes* (Fig. 2a and b, respectively). When the blood samples were stored at 4 °C, viable *E. coli* and *S. pyogenes* were not detectable after 119 and 482 days, respectively. At room temperature, viable *E. coli* was not detectable after 14 days, but *S. pyogenes* was found even after 482 days following a temporary increase.

In the bloodstain samples stored at −30 and −80 °C, there was no significant reduction of *E. coli* and *S. pyogenes* (Fig. 2c and d, respectively). When the bloodstain samples were stored at 4 °C, *S. pyogenes* was found after 482 days, although viable *E. coli* was not detectable after 231 days. At room temperature, both *E. coli* and *S. pyogenes* became undetectable in the bloodstain samples within 119 days.

3.2. Validation of standard DNA

E. coli and *S. pyogenes* DNA samples extracted from 10^6–10^2 cells were used as templates for real-time PCR. Fluorescence was monitored throughout the reaction and the PCR cycle number for each DNA was determined. A standard curve was generated from the PCR cycle number and the log concentration of the *E. coli* and *S. pyogenes* DNA. Quantitation was linear over the range of DNA concentrations examined (data not shown). These bacterial DNA samples were included as standards for each PCR run.

3.3. Quantitation of total bacteria in blood samples by real-time PCR

Before the blood samples were used, K562 DNA was used as a template for real-time PCR to examine human DNA amplification derived from blood. As a result, human DNA was not amplified by PCR using the *E. coli*- or *S. pyogenes*-specific primers (data not shown).

Bacterial DNA extracted at each time point was used as a template and a fragment of approximately 200 or 500 base pairs (bp) was amplified by real-time PCR. The numbers of *E. coli* and *S. pyogenes* in the blood samples were quantified and a change in the number of each bacterium during the period was determined (Fig. 3). There was no significant difference between amplification of the 200- and 500-bp fragments. When the blood samples were stored at −30 and −80 °C, the numbers of

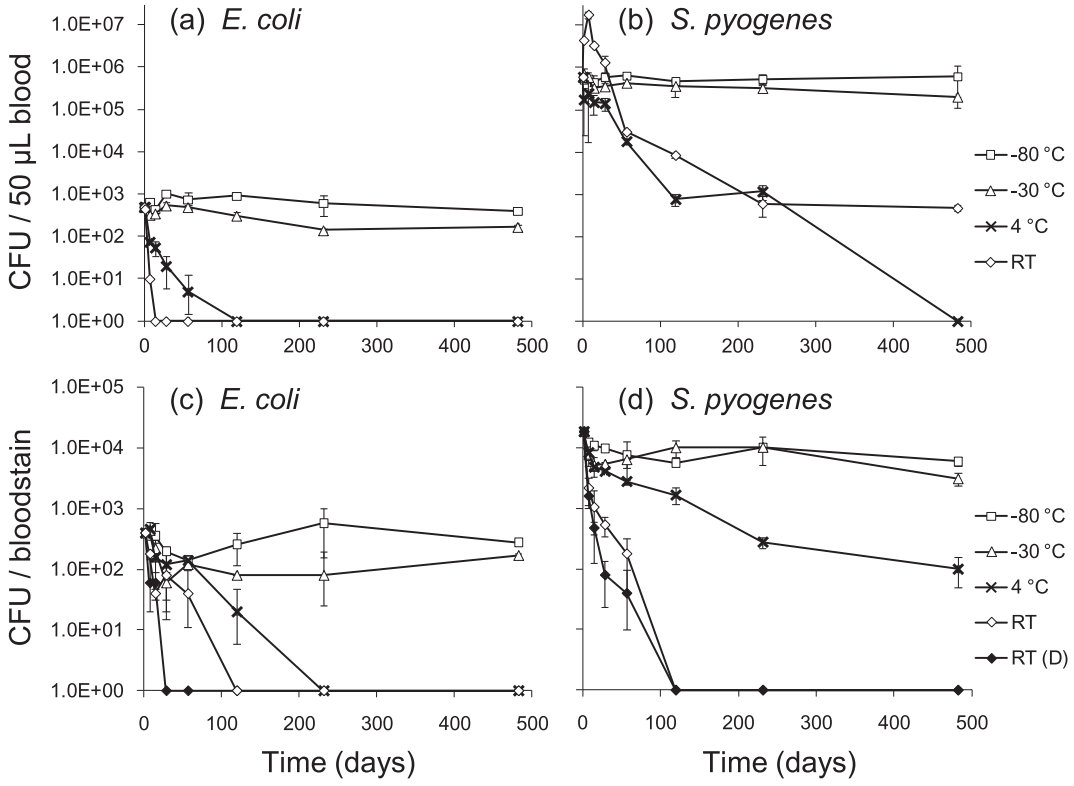

Fig. 2. Population changes of *E. coli* and *S. pyogenes* in the blood (a and b, respectively) and bloodstain (c and d, respectively) samples stored for 482 days. The average of two measurements is plotted to show the surviving number of cells per sample. The error bars represent the standard deviation of two measurements at each time point. RT, room temperature. RT (D), room temperature with desiccant. After the plot reached the horizontal axis, viable bacteria were not detectable.

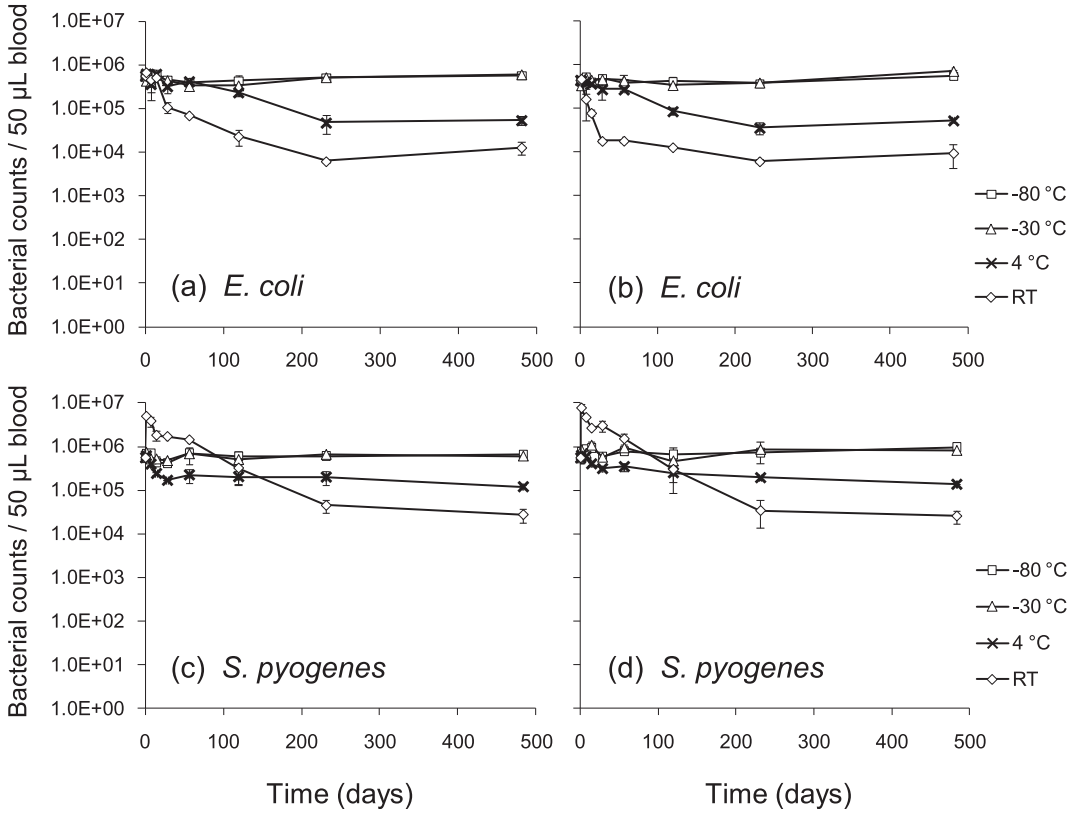

Fig. 3. Changes in the bacterial counts quantified by real-time PCR. Fragments of approximately 200 (a and c) and 500 (b and d) bp were amplified and the numbers of *E. coli* (a and b) and *S. pyogenes* (c and d) in the blood samples were quantified. The average of two quantitative values is plotted and the error bars represent the standard deviation of two measurements at each time point. RT, room temperature.

both *E. coli* and *S. pyogenes* were maintained during the period. In the blood samples stored at room temperature and 4 °C, the bacterial numbers decreased. When the blood samples containing *S. pyogenes* were stored at room temperature, a transient elevation of the total bacterial number was observed along with that of the viable bacterial counts (Fig. 3c and d, respectively).

3.4. Quantitation of total bacteria in bloodstain samples by real-time PCR

Using the same approach as for the blood samples, the numbers of *E. coli* and *S. pyogenes* in the bloodstain samples were quantified by real-time PCR and a change in the number of each bacterium during the period was determined (Fig. 4). There was no significant difference between amplification of the 200- and 500-bp fragments. At each storage condition, there was no significant change in the numbers of both *E. coli* and *S. pyogenes* over the entire storage period.

4. Discussion

Isolation and identification of the bacteria from forensic samples play an important role in solving the biocrime caused by the manner such as injection or food contamination, along with bioterrorism. In addition to straightforward proof of the targeted bacteria, bacterial analysis of forensic samples is also useful for body fluid and feces identification, and providing evidence of death by drowning.[11–13] We prepared the sample model containing bacteria and studied the feasibility of isolation and DNA analysis of the bacteria after long-term storage of the sample.

For the quantitation of total bacteria, fragments of approximately 200 or 500 bp were amplified by real-time PCR to investigate the stability of bacterial DNA. As shown in Figs. 3 and 4, there was no significant difference between amplification of the 200- and 500-bp fragments. Since bacterial DNA of ≥500 bp was not damaged even after 482 days, sequence analysis of bacterial DNA extracted from a specimen stored for a long period is likely to be practicable. In fact, sequencing of the 16S rRNA gene of *S. pyogenes* was performed using DNA from blood samples stored for 231 days at room temperature and −80 °C, and each sequence of the approximately 430-bp fragment was the same as before storage (data not shown).

We initially quantified total bacteria in the blood and bloodstain samples with PCR using 16S rRNA gene-specific primers; however, non-specific amplification was observed in the extraction control sample without bacterial DNA and in the no template control. Contamination of a reagent is detectable by including appropriate negative controls. As this procedure led to inaccurate quantitation, we converted to a procedure using *E. coli*- or *S. pyogenes*-specific primers. Previously, it has been reported that commercial preparations of *Taq* DNA polymerase[14–18] and the DNA isolation kit reagents[19] have been contaminated by exogenous bacterial DNA. When the 16S rRNA gene is used as a target for real-time PCR, digestion of the PCR reagents[20–22] and filtration of the DNA isolation kit reagents[23] are recommended.

For long-term storage, bacterial cultures are usually preserved by ultra-freezing.[24] When blood and bloodstain samples were stored at −30 °C and −80 °C, both viable and total bacterial counts in these samples were maintained during the storage period, indicating that viable cells and bacterial DNA were preserved well. In the blood samples, most of the viable bacteria became

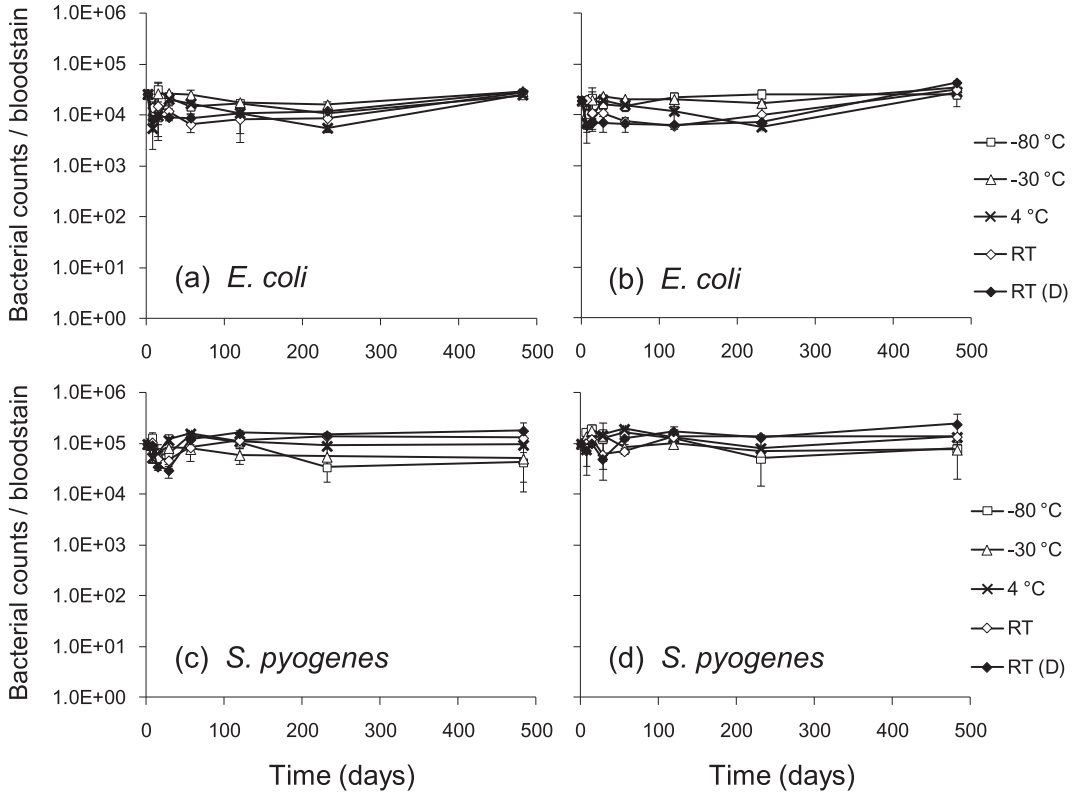

Fig. 4. Changes in the bacterial counts quantified by real-time PCR. The fragments of approximately 200 (a and c) and 500 (b and d) bp were amplified and the numbers of *E. coli* (a and b) and *S. pyogenes* (c and d) in the bloodstain samples were quantified. The average of two quantitative values is plotted and the error bars represent the standard deviation of two measurements at each time point. RT, room temperature. RT (D), room temperature with desiccant.

undetectable when the samples were stored at room temperature and 4 °C. Although viable *S. pyogenes* in the blood samples stored at room temperature was detected even after 482 days (Fig. 2b), this could be due to a starvation response.[25] This is undesirable because such a property may be different from that at the time of storage. Cold conditions such as −30 °C and −80 °C are ideal for the long-term storage of forensic specimens containing bacteria; however, it was still possible to quantify the bacterial DNA of the samples stored at room temperature and 4 °C (Fig. 3). Although most of the viable bacteria became undetectable in the bloodstain samples when stored at room temperature and 4 °C, the total bacterial content of these samples was maintained during the storage period (Fig. 4). This indicates that bacterial DNA in the bloodstain samples stored at room temperature and 4 °C was preserved well, comparable with samples stored at −30 and −80 °C. Drying is commonly used to dehydrate DNA samples.[26] In addition, forensic biological specimens are also often dried to minimize degradation. If a collected specimen cannot be frozen, drying of the specimen should be considered for the analysis of bacterial DNA. Although a desiccant was used for the storage of the bloodstain samples at room temperature, its addition had no significant effect. It would appear that the bloodstain samples were dried well before storage.

In conclusion, we prepared models of blood and bloodstain specimens containing Gram-negative or -positive bacteria and stored them for more than a year, and showed the effect of the storage conditions on viable bacterial and bacterial DNA. This study provides important information for the bacterial analysis of forensic specimens.

Ethical approval

None.

Funding

None.

Conflict of interest

None.

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